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Genetic Diversity within the 3'UTR of the Cynomolgus Macaque (Macaca Fascicularis) LDLR Gene

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Abstract

The low-density lipoprotein receptor gene (LDLR) in body is characterized by several single nucleotide polymorphisms (SNPs) that influence normal variations of plasma lipid profiles. The cynomolgus macaques (Macaca fascicularis) are widely used animals as models for investigating the response of plasma cholesterol to dietary cholesterol, but little is known about genetic variation in its LDLR gene. In this research, genetic diversity in the 3'UTR of LDLR gene was studied using isolated DNAs of 22 cynomolgus macaques which have differences in responsiveness to atherogenic diets.

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The results showed that the 3'UTR sequence of *LDLR* gene had lower genetic diversity (nucleotide composition, number of nucleotide difference, nucleotide diversity, genetic distance, substitution pattern) than human. There are 4 polymorphic sites distributed in 6 haplotypes. Four polymorphic sites were located on *42, *167, *210, and *290. Six identified haplotypes were haplotype I (GGAC), II (GGAT), III (GGGT), IV (AGGT), V (GGGC), and VI (GCGC) with haplotype diversity 0.745% ± 0.064%. Haplotype I – V are parsimony haplotypes, while haplotype VI is singleton haplotype. The obtained polymorphism will become basic information to improve understanding and interpretation of data from hypercholesterolemial studies conducted in this species.

Keywords: 3'UTR; Genetic variations; LDLR gene; Macaca fascicularis.

1. Introduction

The low-density lipoprotein receptor (LDL-R) is a surface cell receptor that plays a crucial role in the receptor-mediated pathway of lipoprotein metabolism [1]. Mutations that render the low-density lipoprotein receptor gene (LDLR) defective cause familial hypercholesterolemia (FH), an inherited autosomal dominant disease with hallmarks of elevated plasma LDL-cholesterol (LDL-C) levels and premature coronary heart disease (CHD). Although most individuals who suffer from CHD do not have FH and their plasma lipoprotein levels are within the reference range, there is an unequivocal evidence that lowering serum cholesterol reduces the risk of CHD events [2]. Thus, because of its pivotal role in lipid metabolisms, a quantitative change of LDLR gene expression is plausibly associated with risk for CHD, and warrants association studies of common genetic variations in non-coding regions of the LDLR gene that may influence its expression at transcriptional or post-transcriptional mRNA levels [3,4]. Several studies have shown associations between genetic variations in the LDLR gene and plasma levels of LDL [5-8]. For example, an association between high plasma LDL-cholesterol levels and the absence of a Pvu II restriction site in intron 15 has been reported in normolipidemic Norwegian and German populations [9-10]. While cynomolgus macaques (Macaca fascicularis) are widely used as models to identify metabolic predictor for hyperresponsiveness to dietary cholesterol and etiology of atherosclerosis in human, it is presently unknown whether they exhibit polymorphisms of similar functional relevance at the LDLR gene.

The human LDLR gene located on chromosome 19 p13.1-3, consist of 18 exons spanning 45 kb, and contains multiple Alu insertions which are involved in several large deletions causing FH [1]. A high Alu density occurs at the 3' untranslated region (3'UTR) of this gene, whose 2.5 kb comprise almost half of the whole mRNA [11]. Several studies showed evidence that the 3'UTR of the LDLR gen can be important site of post-transcriptional regulation [12-13]. Therefore, this research was focused to characterize genetic diversity within the 3'UTR of the LDLR gene in twenty two of cynomolgus macaques which were differences responsiveness to atherogenic diet. The research purposed to identify single nucleotide polymorphism (SNP) within 3'UTR of LDLR gene and its association with responsiveness to atherogenic diet. The results showed that there are 4 polymorphic sites distributed in 6 haplotypes. Four polymorphic sites were located on *42, *167, *210, and *290 and Six identified haplotypes were haplotype I (GGAC), II (GGAT), III (GGGT), IV (AGGT), V (GGGC), and VI (GCGC) with haplotype diversity 0.745% ± 0.064%.

2. Materials and Methods

2.1. Animals

Blood samples were obtained from 22 adult males of cynomolgus macaques (Macaca fascicularis) from captivity in Primate Research Center Bogor Agricultural University, Indonesia. The 22 cynomolgus macaques were grouped based on the responsiveness to atherogenic diet. Used animals and their responsiveness to atherogenic diet shown in Table 1. All treatment procedures applied on the animal had been approved by the Institutional Animal Care and Use Committee (IACUC) with protocol number 12-B009-IR.

Table 1: Animals used in this study and their grouping based on responsiveness to atherogenic diet.

No	Animals (Tatto)	Responsiveness	No	Animals (Tatto)	Responsiveness
1	T3707	Hypo-	12	FE7777	Hyper-
2	K30	Hypo-	13	T3536	Hyper-
3	FC8501	Hyper-	14	C2480	Hyper-
4	T3049	Hyper-	15	T3303	Hyper-
5	FG7998	Hyper-	16	FG7909	Hyper-
6	T3307	Hyper-	17	T3300	Hyper-
7	T3700	Hyper-	18	C0750	Hyper-
8	T3278	Hyper-	19	FC9015	Hyper-
9	FC9113	Hyper-	20	C4927	Hyper-
10	9695	Hyper-	21	C0613	Exstreme
11	C4939	Hyper-	22	T3535	Ekstreme

2.2. Genomic DNA Extraction

Genomic DNA was extracted from all whole-blood samples using the QIAamp™ DNA Mini Kit (QIAGEN, Hilden, Germany) in accordance with manufacturer's instructions

2.3. Primer Design, Amplification, and Sequencing

The primer pairs used for amplification of 3'UTR was designed based on the sequences of the rhesus cynomolgus macaque LDLR gene (http://genome.ucsc.edu, GenBank accession number AY466854) using Primer3 program (http://primer3.wi.mit.edu/). The primers were F: 5'-GAGGGATCAGGATGTGGGAG-3' and R: 5'-ACCACGGATTCAGCCAGATC-3'. Reactions were conducted in a 25 μL volume that containes 5 μL genomic DNA, 1 μL of each primer 10 pmol, 12.5 μL KAPA HotStart ready mix Kit (buffer solution, dNTP and Taq polymerase enzyme) and 5.5 μl nuclease free water. Amplification was performed using a GeneAmp® PCR

System 9700 thermal cycler (Applied Biosystems, Foster City, CA) and the cycling parameters were as follows: denaturation at 94°C for 5 min followed by 40 cycles. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 7 min. The post extention at 25 °C for 4 min. Amplicons were visualized on a transilluminator following agarose gel electrophoresis to check band specificity and sufficiency for subsequent sequence analysis. DNA fragments were then purified using the MinElute Qiagen Kit (QIAGEN, Hilden, Germany) in accordance with manufacturer's instructions, and sequencing was performed in the First BASE Laboratories Sdh Bhd (Malaysia).

2.4. Sequences and Data Analysis

Geneious 7.0.2 (http://geneious.en.softonic.com, the 30-day trial version) was used to edit and assess the quality of sequence data. Consensus sequences were obtained by combining forward and reverse strands for each amplicon and aligning them to the reference sequences of Macaca fascicularis (The Genbank accession number XM_005587996.2). Statistical summaries, including nucleotide composition, number of pairwise differences, nucleotide diversity (π), genetic distance, phylogenetic tree, and pattern heterogeneity were conducted in Mega-6 [14]. Polymorphic sites, gene diversity, and haplotypes analysis were conducted in DnaSP [15]. In silico prediction of transcription factor binding sites at the polymorphic sites located in the 3'UTR was using TFSEARCH (http://www.cbrc.jp/research/db/ TFSEARCH.html).

3. Results

3.1. Amplification and Sequencing of 3'UTR

3'UTR's of the 22 DNA samples of cynomolgus macaques were successfully amplified using forward and reverse primers designed using Primer3 program. The PCR product was in accordance to the expected result, i.e. 750 bp in size (Figure 1). The sequencing results shown that the amplification product consists of 114 bp as part of intron 17, 36 bp was exon 18 and 600 bp was part of 3'UTR located between *1 and *600 positions. The positioning ranges were divided in parallel to the reference in the GenBank (accession number XM_005587996.2), while the numbering was based on the nomenclature proposed by Dunnen dan Antonarakis [16].

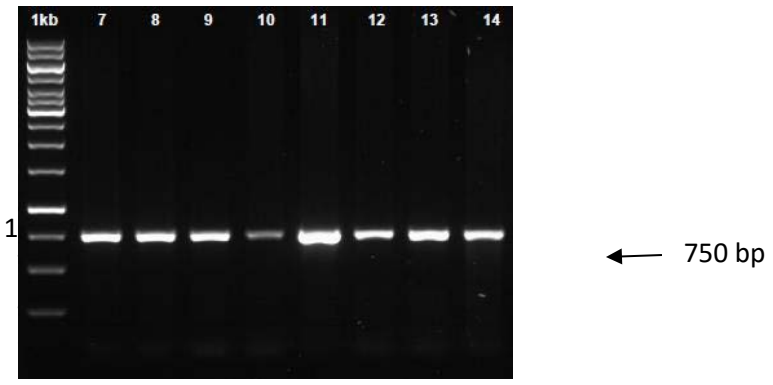

Figure 1: DNA band of 750 bp produced by PCR amplification

3.2. Nucleotide Variations among 3'UTR Sequences

The nucleotide compositions parallelization of the 3'UTR sequences of the 22 blood samples are shown in Table 2. As shown in Table 2, the nucleotide compositions of the 3'UTR sequences were almost equals. The ranges of composition difference in individual nucleotide of each sequence just 0 – 0.2 %.

Table 2: Nucleotide compositions of 3'UTR sequences

Animals (Tattoo)	No. of nucleotides (bp)	Nucleotide compositions (%)			
		T	C	A	G
T4927	600	24,0	27,2	23,8	25,0
FC8501	600	24,2	27,0	23,8	25,0
K30	600	24,2	27,0	23,7	25,2
T3707	600	24,2	27,0	23,8	25,0
T3535	600	24,0	27,2	23,7	25,2
9695	600	24,0	27,2	23,7	25,2
C0613	600	24,0	27,3	23,7	25,0
T3049	600	24,2	27,0	23,7	25,2
T3536	600	24,2	27,0	23,8	25,0
T3700	600	24,2	27,0	23,7	25,2
C4939	600	24,2	27,0	23,8	25,0
T3278	600	24,2	27,0	23,7	25,2
T3303	600	24,2	27,0	23,7	25,2
FC9015	600	24,0	27,2	23,7	25,2
FE7777	600	24,2	27,0	23,7	25,2
T3307	600	24,2	27,0	23,7	25,2
T3300	600	24,0	27,2	23,7	25,2
FG7909	600	24,2	27,0	23,7	25,2
C750	600	24,0	27,2	23,7	25,2
FG7998	600	24,0	27,2	23,7	25,2
C2480	600	24,2	27,0	23,7	25,2
FC9113	600	24,0	27,2	23,7	25,2
Average		24,1	27,1	23,7	25,1

Distance pairwise analysis using number of differences option showed that the the nucleotide differences among the 3'UTR paired sequences ranged from 0 to 3 (Table 3) with an average of 1.017 and a diversity (π) of 0.001696. Sequences having highly nucleotide differences listed in order from the highest: C0613, T3707,

C4939 and T4927, and FC8501 and T3536. The C0613 sequence had 3 nucleotide differences to each of the FC8501, T3707 and T3536 sequences. The T3707 sequence had also 3 nucleotide differences to each of C0613 and T4927 sequences. It was also found that the C4939 sequence had 3 nucleotide differences to each of the C0613 and T4927 sequences. Furthermore, the T4927 had 3 nucleotides lower than that of each of the T3707 and C4939 sequences, and both the FC8501 and T3536 sequences had also 3 nucleotides lower than that belongs to the C0613 one.

Table 3: Matrix of nucleotide differences among 3'UTR paired sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
T4927																						
FC8501	1																					
K30	2	1																				
T3707	3	2	1																			
T3535	1	2	1	2																		
9695	1	2	1	2	0																	
C0613	2	3	2	3	1	1																
T3049	2	1	0	1	1	1	2															
T3536	1	0	1	2	2	2	3	1														
T3700	2	1	0	1	1	1	2	0	1													
C4939	3	2	1	0	2	2	3	1	2	1												
T3278	2	1	0	1	1	1	2	0	1	0	1											
T3303	2	1	0	1	1	1	2	0	1	0	1	0										
FC9015	1	2	1	2	0	0	1	1	2	1	2	1	1									
FE7777	2	1	0	1	1	1	2	0	1	0	1	0	0	1								
T3307	2	1	0	1	1	1	2	0	1	0	1	0	0	1	0							
T3300	1	2	1	2	0	0	1	1	2	1	2	1	1	0	1	1						
FG7909	2	1	0	1	1	1	2	0	1	0	1	0	0	1	0	0	1					
C0750	1	2	1	2	0	0	1	1	2	1	2	1	1	0	1	1	0	1				
FG7998	1	2	1	2	0	0	1	1	2	1	2	1	1	0	1	1	0	1	0			
C2480	2	1	0	1	1	1	2	0	1	0	1	0	0	1	0	0	1	0	1	1		
FC9113	1	2	1	2	0	0	1	1	2	1	2	1	1	0	1	1	0	1	0	0	1	

Genetic distances obtained based on pairwise distance analysis using 2-parameter Kimura model ranged from 0.000 (0%) to 0.005 (0.5%). Identical paired sequences had 0.0% genetic distance, while paired sequence with 3 nucleotide differences had 0.5% genetic distance. The low genetic distance was shown by phylogenetic tree that formed all nodus with bootstrap values less than 75% (Figure 2).

The substitution patterns test observed for the paired sequences using disparity index resulted ini *P* values that

higher than 0.05 (Table 4). The *P* values that higher than 0.05 indicated that there no differences in substitution patterns observed for the paired sequences (homogeneity in substitution patterns). This meant that nucleotide differences occurred among the paired sequences were insignificant.

Table 4: Homogeneity test of substitution patterns among 3'UTR paired sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
T4927																						
FC8501	1,000																					
K30	1,000	1,000																				
T3707	1,000	1,000	1,000																			
T3535	1,000	1,000	1,000	1,000																		
9695	1,000	1,000	1,000	1,000	1,000																	
C0613	1,000	1,000	0,410	1,000	1,000	1,000																
T3049	1,000	1,000	1,000	1,000	1,000	1,000	0,438															
T3536	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000														
T3700	1,000	1,000	1,000	1,000	1,000	1,000	0,436	1,000	1,000													
C4939	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000												
T3278	1,000	1,000	1,000	1,000	1,000	1,000	0,426	1,000	1,000	1,000	1,000											
T3303	1,000	1,000	1,000	1,000	1,000	1,000	0,436	1,000	1,000	1,000	1,000	1,000										
FC9015	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000									
FE7777	1,000	1,000	1,000	1,000	1,000	1,000	0,396	1,000	1,000	1,000	1,000	1,000	1,000	1,000								
T3307	1,000	1,000	1,000	1,000	1,000	1,000	0,458	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000							
T3300	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000						
FG7909	1,000	1,000	1,000	1,000	1,000	1,000	0,426	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000					
C0750	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000				
FG7998	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000			
C2480	1,000	1,000	1,000	1,000	1,000	1,000	0,418	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000		
FC9113	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	

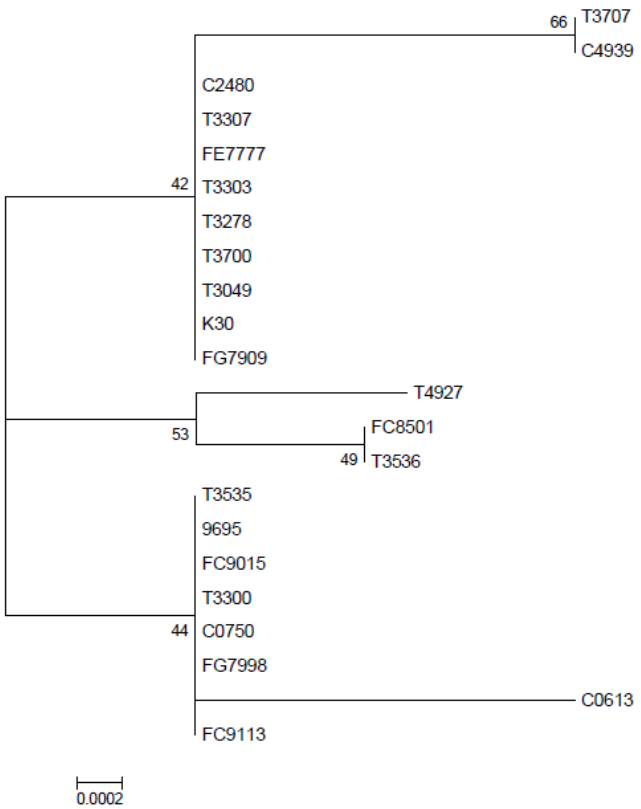

Figure 2: Phylogenetic tree of 3'UTR sequences using Neighbor Joining with 1000 times bootstrap. Values on each branch of the tree indicate the bootstrap values.

3.3. Single Nucleotide Polimorphism and Haplotype

3'UTR sequences analysis of the 22 cynomolgus macaques identified 6 haplotypes from 4 single nucleotide polymorphisms (SNPs) at *42, *167, *210, and *290 positions (Table 5). Polymorphic sites consisted of tree parsimony variable sites (*42, *210, and *290) and one singleton variable (*167). Results of TFSEARCH program revealed no binding site of transcription factors within or in the vicinity of the polymorphic sites indicating that the identified polymorphisms were nonfunctional. A Functional polymorphism is defined as a site that causes binding site appearance or disappearance of new transcription factors. Likewise, the haplotype products are non-functional [17].

The six identified haplotypes consisted of 5 common haplotypes, i.e. haplotypes I (GGAC), II (GGAT), III (GGGT), IV (AGGT), and V (GGGC), and 1 singleton haplotype (specific), i.e. haplotype VI (GCGC) with diversity of 0.745 ± 0.064. The haplotype V was similar to the reference haplotype of cynomolgus macaques in the GenBank. The Haplotype III contained the highest number of individuals, i.e. nine individuals, whilst the haplotypes I and V had the least, i.e. one individual each (Table 5). The common haplotypes performed responsiveness variations as hypo-responsiveness, hyper-responsiveness, and extreme, whilst the specific haplotype VI (GCGC) that belonged only to C0613 individual showed extreme responsiveness to atherogenic diet (Table 1).

Table 5: Identified SNP in 3'UTR of *LDLR* gene that in parallel to the GenBank reference (accession number XM_005587996.2)

Haplotypes	Position whitin the 3'UTR				No. of individuals	No. of tattoo
	*42	*167	*210	*290		
Ref	G	G	G	C	-	-
I	G	G	A	C	1	T4927
II	G	G	A	T	2	FC8501, T3536
III	G	G	G	T	9	K30, T3049, T3700, T3278, T3303, FE7777, T3307, FG7909, C2480
IV	A	G	G	T	2	T3707, C4939
V	G	G	G	C	7	T3535, 9695, FC9015, T3300, C0750, FG7998, FC9113
VI	G	C	G	C	1	C0613

4. Discussion

The 3'UTR sequences have long been recognized as a regulatory region that is important for the appropriate expression of many genes [18-19]. It contains *cis*-elements that affect the stability of transcripts, and/or influence the translation by interacting with the 5' sequence of the transcripts. For example, a stretch of AU-rich

element (ARE) containing an AUUUA penta-nucleotide motif serves as a recognition site for specific proteins that increase the rate of poly A tail shortening and mRNA degradation. The 3'UTR of the human LDLR genes includes three regions each containing an ARE, and Wilson et al. [20] demonstrated that each element shortened the half-life of the reporter gene transcript by approximately 30% when introduced into the 3'UTR of the β globin gene in a tissue culture based system. Furthermore, previously study shown that the removal of two of the three AREs in the 3'UTR of the LDLR gene leads to more stable transcripts and increased steady state levels of LDLR mRNA in mice [21]. Therefore, common SNPs within or near these cis-elements may affect the binding of proteins to ARE or other control elements and hence alter gene expression in general human populations.

Overall, the 3'UTR sequences of LDLR gene of the cynomolgus macaques indicated the low genetic diversities (average of nucleotide differences, nucleotide diversity, genetic distances, and substitution patterns). The nucleotide diversity (π) is an accurate parameter in order to describe the genetic diversities. Positive elements using nucleotide diversity (π) are independent on the sample sizes as well as on the DNA's lengths [22-23]. This means that the probability that small sample sizes may extensively depict the actual conditions of population. The nucleotide diversity (π) obtained in this research (0.001696) is far less than the nucleotide diversity (π) of 4 country populations, Africa, Asia, Caucasus, and America, each has π of 0.422 ± 0.046, 0.545 ± 0.029, 0.503 ± 0.027 and 0.492 ± 0.011 respectively [24].

The number of SNPs and haplotypes founded in this research were smaller than that of reported in human body. As reported by Fagundes et al. [24] that there were 21 polymorphic sites distributed within 15 haplotype observed in 111 3'UTR sequences of LDLR gene (with 784 nucleotide numbers) of 4 country's populations. Meanwhile, the haplotype diversities were still in the range of haplotype diversities of the 4 country's populations i.e. 0.751 – 0.860. The SNPs and haplotypes are common genetic variations used as genetic markers for normal profile variations of lipid plasma. In human, 2 SNPs within 3'UTR have been reported to be potential as the lipid response markers for certain populations; the Caucasus population [25]. Also, the presence of 1 haplotype out of 6 haplotypes produced by 5 polymorphic sites which related to lowering lipid response caused by introduction of simvastatin [26].

The presence of haplotype VI (GCGC) associated with extreme responsiveness to the atherogenetic diet indicates that the 3'UTRs in cynomolgus macaques have also common genetic associated with response variations to the atherogenic diet that has not been published previously. Although these results are potentially important, they must be interpreted with caution until confirmed. The association of GCGC specific haplotype within 3'UTR with extreme responsiveness of cynomolgus macaques to the atherogenetic diets has promoted the SNP g.*167G>C as being potentially used as genetic markers. The presence of the non functional SNP g.*167G>C indicates the possibility of the SNP located in disequilibrium linkage with other SNPs in the same gene or in different gene that are functional to extremely increase the cholesterol plasma when atherogenetic diets are given.

Generally, SNPs or haplotypes identifications in cynomolgus macaques are mainly related to the origin or geographical distributions [27-29]. Investigations on individual variations that affect susceptibilities or other

defects are very view compared to biomedical researches on cynomolgus macaques. Some of them are genetic variations against malaria susceptibility [17], drug safety [30] and neurobiology reactivity due to stress [31]. Identification of SNPs within the 3'UTR of LDLR gene as genetic markers of responsiveness is an important advancement in the investigations using cynomolgus macaques as models in order to understand the atherosclerosis. These are due to most of researches conducted using animals to investigate the responsiveness to the atherogenetic diets is not yet based on the appropriate genetic variability.

Differences in the results of toxicology and pharmacokinetic investigations on cynomolgus macaques [32-34] might be related to the genetic diversity. Therefore, researches conducted to know the individual genetic diversity is expected being able to explain the variability phenomena. This research is an initial stage as to understand the genetic diversity of the cynomolgus macaque LDLR gene. Researches on genetic diversity of the LDLR gene is still needed to be conducted in other regions in order to know other genetic variations and their associations with the responsiveness to atherogenic diets.

5. Conclusion

The research revealed that the 3'UTR of *LDLR* gene of cynomolgus macaques had genetic diversity levels that were lower than the ones belong to human being. Four identified polymorphic sites distributed in six haplotypes indicated the presence of genetic variation that potentially be used as genetic markers for responsiveness of cynomolgus macaques to the atherogenic diets.

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